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(71) Applicant: **INBICON A/S** [DK/DK]; Kraftværksvej 53, DK-7000 Fredericia (DK).

(72) Inventors: **LARSEN, Jan**; Østerbro 28, DK-5690 Tommerup (DK). **JEPPESEN, Martin Dan**; Ejstrupvej 40, DK-5200 Odense V (DK). **MOGENSEN, Kit Kellebjerg**; Kraborrevej 4, DK-7000 Fredericia (DK).

(74) Agent: **PLOUGMANN & VINGTOFT A/S**; Rued Langgaards Vej 8, DK-2300 Copenhagen S (DK).

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(54) Title: METHODS OF PROCESSING EMPTY FRUIT BUNCHES (EFB) TO FERMENTABLE SUGARS USING MULTIPLE-STAGE ENZYMATIC HYDROLYSIS

(57) Abstract: Provided are methods of processing empty fruit bunches (EFB) using cellulase enzymatic hydrolysis in multiple stages. Hydrolysis yields are improved in a multiple-stage process featuring at least one solid/liquid separation step to an extent that is surprisingly great, much more than can be accounted for by alleviation of glucose product inhibition of cellulase enzymes.

Methods of processing empty fruit bunches (EFB) to fermentable sugars using multiple-stage enzymatic hydrolysis.

Field: The invention relates to methods of processing oil palm empty fruit bunches (EFB) to fermentable sugars using enzymatic hydrolysis.

Inventors: Martin Dan Jeppesen, Kit Mogensen and Jan Larsen

Background.

Historical reliance on petroleum and other fossil fuels has been associated with dramatic and alarming increases in atmospheric levels of greenhouse gases. International efforts are underway to mitigate greenhouse gas accumulation, supported by formal policy directives in many countries. One central focus of these mitigation efforts has been development of processes and technologies for utilization of renewable plant biomass to replace petroleum as a source of precursors for fuel and other chemical products. The annual growth of plant-derived biomass on earth is estimated to approximate 1 x 10^11 metric tons per year dry weight. (Lieth and Whittaker 1975) Biomass utilization is, thus, an ultimate goal in development of sustainable economy.

Fuel ethanol produced from sugar and starch based plant materials, such as sugarcane, root and grain crops, is already in wide use, with global production currently topping 73 billion liters per year. (Renewable Fuels Association statistics, 2010). Ethanol has always been considered an acceptable alternative to fossil fuels, being readily usable as an additive in fuel blends or even directly as fuel for personal automobiles. However, use of ethanol produced by these "first generation" bioethanol technologies does not actually achieve dramatic reduction in greenhouse gas emission. The net savings is only about 13% compared with petroleum, when the total fossil fuel inputs to the final ethanol outputs are all accounted. (Farrell et al 2006) Moreover, both economic and moral objections have been raised to the "first generation" bioethanol market. This effectively places demand for crops as human food into direct competition with demand for personal automobiles. And indeed, fuel ethanol demand has been associated with increased grain prices that have proved troublesome for poor, grain-importing countries.

Accordingly, great interest has arisen in so-called "second generation" bioethanol, produced from lignocellulosic biomass such as crop wastes (stalks, cobs, pits, stems, shells, husks, etc...), grasses, straws, wood chips, waste paper and the like. In "second generation" technology,

fermentable 6-carbon and 5-carbon sugars are liberated from biomass polysaccharide polymer chains by enzymatic hydrolysis or, in some cases, by pure chemical hydrolysis. The fermentable sugars obtained from biomass conversion in a "second generation" biorefinery can be used to produce fuel ethanol or, alternatively, other fuels such as butanol, or lactic acid monomers for use in synthesis of bioplastics, or many other products.

For biomass processing that relies on enzymatic hydrolysis, some pretreatment is typically required, because of limitations of lignocellulose' physical structure. A wide variety of different pretreatment schemes have been reported. From an environmental and "renewability" perspective, hydrothermal pretreatments are especially attractive. These utilize pressurized steam/liquid hot water at temperatures on the order of 160 – 230 o C to gently melt hydrophobic lignin that is intricately associated with cellulose strands, to solubilize a major component of hemicellulose, rich in 5 carbon sugars, and to disrupt cellulose strands so as to improve accessibility to productive enzyme binding.

Enzyme preparations that can "saccharify" pretreated lignocellulosic feedstocks have recently emerged as a new commercial market. Enzymatic hydrolysis of pretreated biomass, that is, enzymatic hydrolysis of cellulose to glucose and of hemicellulose to constituent oligo- and monosaccharides is typically conducted using a mixture of different hydrolytic enzyme activities. As is well known in the art, many fungi and bacteria have been identified which degrade lignocellulosic biomass in nature. Preparations of enzymes obtained from such organisms typically provide a variety of different enzyme activities that, collectively and synergistically, provide effective hydrolysis of lignocellulosic materials. Such enzyme preparations are typically termed "cellulase" preparations, since they comprise cellulytic enzymes that catalyse hydrolysis of glucosidic linkages in cellulose, including endoglucanases, which introduce nicks in the cellulosic polymer chain thereby exposing reducing ends, and cellobiohydrolases or exoglucanases, which catalyze from reducing and non-reducing ends release of oligosaccharide products from the cellulosic polymer chain. Such preparations further typically comprise B-glucosidases, which catalyse hydrolysis of oligosaccharide products to fermentable monosaccharides as well as a variety of hemicellulases, including endoxylanases, exoxylanases, exoxylosidases, mannosidases, acetyl xylan esterases, and other activities. For review see (Rabinovich et al., 2002; Taherzadeh and Karimi, 2007).

Enzyme preparations comprising a multi-enzyme mixture sufficient to "saccharify" lignocellulosic biomass may be obtained by a variety of methods known in the art from a variety of

microorganisms, including at least bacteria such as species of *Clostridium, Cellumonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Mocrobispora,* and *Streptomyces* and at least fungi such as species of *Trichoderma, Penicillium, Fusarium, Humicola, Aspergillus* and *Phanerochaete*.

Commercially available cellulase preparations are now offered that have been specifically optimized for lignocellulosic biomass conversion. The term "commercially available cellulase preparation" refers to a mixture of enzyme activities that is sufficient to provide enzymatic saccharification of pretreated lignocellulosic biomass and that comprises cellulase, xylanase and B-glucosidase activities. The term "optimized for lignocellulosic biomass conversion" refers to a product development process in which enzyme mixtures have been selected and modified for the specific purpose of improving hydrolysis yields and/or reducing enzyme consumption in hydrolysis of pretreated lignocellulosic biomass to fermentable sugars. Selection and modification of enzyme mixtures may include genetic engineering techniques, for example such as described in (Zhang et al., 2006) or by other methods known in the art.

Commercially available cellulase preparations optimized for lignocellulosic biomass conversion are typically identified by the manufacturer and/or purveyor as such. These are typically distinct from commercially available cellulase preparations for general use or optimized for use in production of animal feed, food, textiles detergents or in the paper industry.

In our experience with large scale processing of lignocellulosic biomass, we have seen excellent results using commercially available cellulase preparations optimized for lignocellulosic biomass applied to hydrothermally pretreated feedstocks such as wheat straw, bagasse and corn stover. However, using pretreated oil palm empty fruit bunches (EFB), we have seen anomalously low hydrolysis yields using the same commercially available enzyme preparations that proved highly satisfactory with other feedstocks.

Here we report that, in the particular case of EFB, hydrolysis yields can be substantially improved by the simple expedient of conducting enzymatic hydrolysis in at least two distinct stages, with solid/liquid separation between stages followed by re-suspension of the solid residual for further enzymatic hydrolysis.

Brief description of the figures.

Figure 1 shows the effect on hydrolysis yield of washing pretreated wheat straw and EFB to remove cellulase inhibitors prior to hydrolysis using a commercially available cellulase preparation optimized for lignocellulosic biomass conversion.

Figure 2 shows a comparison of hydrolysis yields obtained from pretreated washed EFB using a commercially available cellulase preparation optimized for lignocellulosic biomass conversion (A) in a two-stage hydrolysis process and in a single-stage hydrolysis process with equivalent water content, and (B) in a two-stage hydrolysis process with enzyme supplementation in the second stage and in a single-stage hydrolysis process with equivalent water content.

Figure 3 shows the effect on hydrolysis yields obtained from pretreated washed EFB using a commercially available cellulase preparation optimized for lignocellulosic biomass (A) in the presence of liquid hydrolysate obtained from the first stage of a two-stage hydrolysis process, and (B) in the presence of liquid hydrolysate obtained from the second stage of a two-stage hydrolysis process.

Figure 4 shows release of enzyme protein to solution as a function of degree of hydrolysis of pretreated wheat straw using a commercially available cellulase preparation optimized for lignocellulosic biomass conversion.

Detailed description of embodiments.

End-product inhibition by glucose and cellobiose of enzyme-catalysed cellulose hydrolysis was previously considered a significant problem in enzymatic biomass processing. One previously reported solution to this perceived problem was the use of multiple-stage enzymatic hydrolysis. Where partial hydrolysis was conducted in a first stage, followed by solid/liquid separation and re-suspension of the solid residual in fresh aqueous medium, the enzymatic hydrolysis reaction in the second or other subsequent stages experienced a diminished concentration of accumulated glucose. Accordingly, glucose inhibition could be minimized by conducting hydrolysis in multiple stages. See US 2010/0068768 and see (Yang et al. 2010).

The component enzymes of cellulase preparations used for multiple-stage hydrolysis as previously reported typically required some manipulation. One important component of such preparations was beta-glucosidase activity, which catalyses the hydrolysis of the soluble disaccharide cellobiose to glucose monomers. Because beta-glucosidase activities were always soluble, the enzyme

activity would typically follow the liquid fraction during solid/liquid separation. Accordingly, some modification of beta-glucosidases so that they would bind to the lignin-rich solid residual (US 2010/0068768) or some recovery of soluble enzymes (Yang et al. 2010) was previously considered necessary for multiple-stage hydrolysis.

The two companies which offer commercial cellulase preparations optimized for lignocellulosic biomass conversion, NOVOZYMES Tm and GENNENCOR Tm, have by now modified their enzyme preparations such that end-product inhibition by glucose is no longer particularly significant. The current "generation" of commercially available cellulase preparations optimized for lignocellulosic biomass conversion, which include the preparation offered by NOVOZYMES Tm under the trade name CELLIC CTEC3 Tm and the preparation offered by GENNENCOR Tm under the trade name ACCELLERASE TRIO Tm, show no significant inhibition of cellulase activity in the presence of 40 g/L glucose (data not shown but see ACCELLERASE TRIO Tm Product Information Sheet, Danisco Inc. 2011 and see CELLIC CTEC3 Tm Application Sheet, which are hereby incorporated by reference in entirety). One skilled in the art will certainly anticipate that future "generations" of commercially available cellulase preparations optimized for lignocellulosic biomass conversion will be at least as insensitive to glucose inhibition as is the current "generation."

Multiple-stage hydrolysis is counter-indicated for enzymatic hydrolysis reactions using current or better "generations" of commercially available cellulase preparations optimized for lignocellulosic biomass conversion, where these preparations exhibit substantially reduced susceptibility to glucose end-product inhibition of catalysis.

We previously considered whether multiple-stage hydrolysis might be effective as a means of counteracting the negative effects of soluble cellulase inhibitors produced as byproducts of hydrothermal pretreatment, including acetate, phenolic compounds and xylooligosaccharides. For review on pretreatment-derived cellulase inhibitors see Duarte et al 2012.

Using hydrothermally pretreated wheat straw as a representative lignocellulosic biomass, we determined hydrolysis yields as a function of % removal of dissolved solids from the pretreated "solid" fraction in a hydrolysis reaction conducted at 20% dry matter (DM). The term "dry matter" refers to total dissolved and undissolved solids. Hydrolysis was conducted using a current "generation" commercially available cellulase preparation optimized for lignocellulosic biomass conversion (the preparation offered by NOVOZYMES Tm under the trade name CELLIC CTEC3

Tm). Results are shown in Figure 1. The maximal improvement in hydrolysis yields which could be achieved by washing to remove soluble cellulase inhibitors was at most about 8% in absolute terms, where "% yield" is expressed as percent theoretical yield, calculated as a percentage of the amount of glucose that could theoretically be obtained based on the cellulose content of the material assuming 1.11 g glucose per g cellulose. If instead of washing, a two-step hydrolysis process were to be used to remove soluble cellulase inhibitors, the maximal removal of dissolved solids which could be achieved in the solid/liquid separation step following a first partial initial hydrolysis stage would be a function both of the dry matter content of the partial initial hydrolysis reaction and also of the extent to which the solid residual from the partial initial hydrolysis reaction could be pressed to a high dry matter content. For example, in a partial initial hydrolysis reaction at 20% DM, where the residual solid is pressed to 40% DM and then resuspended for a second stage hydrolysis reaction at 20% DM, it would be expected that about 63% of dissolved solids could be removed for the second hydrolysis stage. In the case of wheat straw, this would be expected to provide, at the very best, on the order of 4% improvement in hydrolysis yields in absolute terms *only in the second hydrolysis reaction*. This very slight effect would not likely prove worthwhile to justify the process complexity of multiple-stage hydrolysis on production scale.

Moreover, in actual practice, two-step hydrolysis as a means of overcoming negative effects of soluble cellulase inhibitors generated by pretreatment typically produces less than maximal improvements, perhaps due to loss of enzyme activity during transfer to the second stage, or for other reasons not currently understood. We previously compared hydrolysis yields obtained with pretreated wheat straw in a 20% DM two-step reaction with yields obtained in a 20% DM one-step reaction to which a volume of additional water was added equivalent to that used for resuspension of the residual solid in the second hydrolysis stage. The results (not shown) obtained using an earlier "generation" commercially available cellulase preparation optimized for lignocellulosic biomass conversion (the preparation offered by NOVOZYMES Tm under the trade name CELLIC CTEC2 Tm) indicated that two-step hydrolysis does not appreciably relieve the negative effects of soluble cellulase inhibitors with wheat straw.

Thus, multiple-stage hydrolysis is also counter-indicated as a means of avoiding negative effects of soluble cellulase inhibitors, for enzymatic hydrolysis reactions using current or better "generations" of commercially available cellulase preparations optimized for lignocellulosic biomass conversion. The susceptibility of cellulase preparations to inhibition by soluble cellulase inhibitors generated during pretreatment is, in any case, expected to be diminished in future generations of

commercially available cellulase preparation optimized for lignocellulosic biomass conversion. See e.g. Zhang et al 2012.

However, unexpectedly, we have determined that multiple-stage hydrolysis provides a very significant enhancement of hydrolysis conversion yields in the particular case of hydrothermally pretreated EFB. We compared hydrolysis yields obtained in an 18% DM two-step reaction using hydrothermally pretreated and washed EFB with yields obtained in an 18% DM one-step reaction to which a volume of additional water was added equivalent to the volume used for resuspension of the residual solid in the second hydrolysis stage. The results obtained using a current "generation" commercially available cellulase preparation optimized for lignocellulosic biomass conversion (the preparation offered by GENNENCOR Tm under the trade name ACCELLERASE TRIO Tm) are shown in Figure 2. As shown, the simple expedient of two-step hydrolysis in the particular case of EFB gives rise to a very large increase in conversion yield on the order of 13% in actual terms. This enhancement is much larger than the expected effect of washing to remove soluble cellulase inhibitors, and certainly very much larger than the expected effect of two-stage hydrolysis to remove soluble cellulase inhibitors.

The extraordinary effect of two-step hydrolysis using EFB cannot be explained by removal of any soluble cellulase inhibitor generated by pretreatment. We compared hydrolysis yields obtained in an 18% DM one-step reaction with hydrothermally pretreated EFB which had been variously washed to remove either 70% or 97% of dissolved solids. Washing so as to remove 97% dissolved solids was calculated to provide an equivalently washed control for the two-step hydrolysis experiment. The results obtained using a current "generation" commercially available cellulase preparation optimized for lignocellulosic biomass conversion (the preparation offered by GENNENCOR Tm under the trade name ACCELLERASE TRIO Tm) are shown in Figure 1. As shown, with hydrothermally pretreated EFB, this difference in degree of washing did not provide any enhancement of conversion yields. Accordingly, the enhancement provided in the two-step hydrolysis apparently cannot be explained by removal of soluble cellulase inhibitors produced by pretreatment. Without wishing to be bound by theory, we consider that the enhancement of conversion yields with EFB using two-step hydrolysis can possibly be explained by the release *during the course of the hydrolysis reaction* of some as-yet-uncharacterized inhibitor of one or more enzyme activities comprising the commercially available cellulase preparation optimized for lignocellulosic biomass conversion. Such an inhibitor could conceivably be a hemicellulose polysaccharide fragment or a lignin-derived compound. EFB is known to possess some unusual

compositional features, including a very high relative percentage of acid-soluble lignin. See e.g. WO2011002329.

Conveniently, the residual solid remaining after partial initial hydrolysis in a multiple-stage hydrolysis scheme will typically retain beta glucosidase activities as well as all other enzyme proteins that comprise current “generations” of commercially available cellulase preparations optimized for lignocellulosic biomass conversion. We have previously determined that a previous “generation” of commercially available cellulase preparations optimized for lignocellulosic biomass conversion, which include the preparation offered by NOVOZYMES Tm under the trade name CELLIC CTEC2 Tm and the preparation offered by GENNENCOR Tm under the trade name ACCELLERASE 1500 Tm, comprise modified beta glucosidase activities and other enzyme proteins configured such that effectively all enzyme proteins will adhere to the solid fraction during solid/liquid separation. (Data not shown).

Accordingly, in some embodiments, the invention provides a method for processing empty fruit bunches (EFB) to fermentable sugars comprising
- providing EFB feedstock,
- pre-treating the feedstock using hydrothermal pre-treatment,
- subjecting the pretreated feedstock to partial enzymatic hydrolysis using a cellulase preparation,
- separating the liquid hydrolysate from the solid residual obtained from partial hydrolysis, and
- resuspending the solid residual from partial hydrolysis and continuing enzymatic hydrolysis.

"Hydrothermal pretreatment" refers to the use of water, either as hot liquid, vapour steam or pressurized steam comprising high temperature liquid or steam or both, to "cook" biomass, at temperatures of 120o C or higher, either with or without addition of acids or other chemicals. The pH at which biomass is hydrothermally pretreated refers to the pH of the wetted biomass/biomass slurry as it enters a pretreatment reactor. The total solids content at which biomass is hydrothermally pretreated refers to the combined w/w percentage of water insoluble and water soluble solids present in the wetted biomass/biomass slurry as it enters a pretreatment reactor.

"Partial initial hydrolysis" refers to an enzymatic hydrolysis reaction in which cellulase, B-glucosidase and hemicellulase activities are used to hydrolyse biomass to a percent conversion that is 80% or less followed by solid/liquid separation and resuspension of the solid residual in one or more subsequent hydrolysis steps.

The "liquid hydrolysate" obtained after partial initial hydrolysis refers to the liquid fraction remaining after solid/liquid separation of the partial initial hydrolysis mixture.

The "solid residual" obtained from partial initial hydrolysis refers to the solid fraction remaining after solid/liquid separation of the partial initial hydrolysis mixture. As will be readily understood by those skilled in the art, the "solid residual" will typically comprise considerable liquid content, where the total solids content of the solid residual is typically within the range of 20 to 50%. The solids content of the solid residual comprises primarily lignin and unhydrolysed cellulose and hemicelluloses although some water soluble solids are typically also included.

"Subsequent hydrolysis step" refers to a continued enzymatic hydrolysis of biomass that has been subject to partial initial hydrolysis. The % conversion achieved in subsequent hydrolysis steps refers to the cumulative % conversion of the biomass over initial partial hydrolysis and any number of subsequent hydrolysis steps.

The "liquid fraction obtained after pretreatment" refers to the liquid phase of pretreated biomass as it exists immediately after pretreatment and after equilibration to 25o C and atmospheric pressure. It will be readily understood that "liquid fraction" exists whether or not any physical solid/liquid separation step is used. Liquid fraction comprises primarily water and water soluble material.

The term “% conversion” as used herein refers to conversion of cellulose into glucose. The term "% conversion" refers to % of the amount that could theoretically be obtained based on the cellulose content of the material. 100% theoretical recovery of glucose from cellulose is 1.110 g glucose per g cellulose.

Any suitable EFB feedstock may be used. In some embodiments, EFB feedstock may be used in the form in which it exists after palm oil processing. In some embodiments, EFB may be subject to methods for removal of lipid-soluble substances and/or lignin including initial hot water treatment or to any of the methods described in WO2011002329, which is hereby incorporated by reference in entirety. In some embodiments, EFB or EFB previously subject to some initial thermal or chemical processing, may be subject to milling, grinding, shredding or other forms of particle size reduction.

EFB feedstock is subject to hydrothermal pretreatment, typically within a pressurized pretreatment reactor.

The "severity" of hydrothermal pretreatment refers to the harshness of conditions to which biomass feedstock has been subject. A variety of different composite parameters have been proposed that provide a scalar index by which different pretreatments schemes may be compared. Classic "severity, " Ro, is defined as (residence time in minutes) x (EXP[(pretreatment temperature - 100) / 14.75]) and is typically referred to as a logarithmic value, log Ro. Many hydrothermal pretreatment schemes such as dilute acid pretreatment and acidic steam explosion require relatively strong acid conditions but can typically be practiced at lower temperatures. Thus, a second severity parameter, Ro', is often used which includes a pH dimension such that, expressed as a log value, log Ro' is simply log Ro - pH. Some hydrothermal and other pretreatment schemes depend on relatively high pH conditions, such as ammonia fiber explosion and alkaline wet oxidation. Accordingly, still another severity parameter, Ro", is reported which accounts for the pH dimension such that log Ro" is log Ro + pH.

Alternatively, pretreatment severity can be expressed in terms of residual xylan content. The term “xylan number” refers to residual xylan content measured as follows: Pretreated biomass is subject to solid/liquid separation to provide a solid fraction at 30% total solids which is swelled with liquid fraction obtained after pretreatment. This solid fraction is then mixed with water in the ratio of total solids to water of 1:3. The solid fraction washed in this manner is then pressed to 30% total solids. Xylan content of the solid fraction washed in this manner is determined using the method of A. Sluiter, et al., "Determination of structural carbohydrates and lignin in biomass," US National Renewal Energy Laboratory (NREL) Laboratory Analytical Procedure (LAP) with issue date April 25, 2008, as described in Technical Report NREL/TP-510-42618, revised April 2008, which is expressly incorporated by reference herein in entirety.

In the case of hydrothermal pretreatments, it is well known in the art, and has been widely discussed, that pretreatment severity must be optimized between conflicting tendencies. Severity should be sufficiently high so as to open lignocellulose structure for productive enzyme binding and thereby achieve reasonable conversion yields from the enzymatic hydrolysis reaction. Yet severity should also be sufficiently low so as to minimize loss of C5 sugars from hemicellulose and the associated increased production of byproducts of the pretreatment reaction that can inhibit fermentive organisms.

Hydrothermal pretreatment may be conducted by a variety of methods well known in the art. Steam pretreatment typically may be conducted either as a "steam explosion" or using high pressure steam without explosive release of pretreated biomass. Steam pretreatment is typically

conducted at high temperatures, between 170 and 220° C, and at high pressures, between 4 and 20 bar, where water exists as a mixture of liquid and vapour. In some embodiments, lignocellulosic biomass is pretreated by hydrothermal pretreatment at temperatures between 170 and 200° C. In some embodiments, biomass feedstocks may be subject to particle size reduction and/or other mechanical processing such as grinding, milling, shredding, cutting or other processes prior to hydrothermal pretreatment. In some embodiments, biomass feedstocks may be washed and/or leached of valuable salts prior to pretreatment, as described in Knudsen et al. 1998. ("Possibilities and evaluation of straw pretreatment" presented at the 10th european biomass conference in Würzburg in 1998. Authors are N.O. Knudsen, P.A. Jensen, B. Sander and K. Dam-Johansen.). It is advantageous to conduct pretreatment at the highest possible levels of total solids that can be used, without resulting in diminished ultimate hydrolysis efficiency.

In some embodiments, hydrothermal pretreatment is conducted without supplemental oxygen as required for wet oxidation pretreatments, or without addition of organic solvent as required for organosolv pretreatment, or without use of microwave heating as required for microwave pretreatments. In some embodiments, hydrothermal pretreatment is conducted at temperatures of 140o C or higher, or at 150o C or higher, or at 160o C or higher, or 220o C, or lower.

Hydrothermal pretreatments may be conducted at different degrees of wetting, i.e., at different "total solids" content. The raw biomass feedstock comprises some quantity of water as well as a "dry weight" that remains after all water is removed. Total solids refers to both water insoluble solids and water soluble solids.

The total solids content of hydrothermally pretreated biomass comprises both a liquid component (hereafter "liquid fraction") and a water insoluble "fiber" component. The "fiber" component comprises insoluble solids that are swelled with "liquid fraction." In some hydrothermal pretreatments such as steam explosion methods, the "fiber" component comprises insoluble solids that do not actually retain fibrous structure *per se*. The "liquid fraction" comprises solubilized hemicellulose, comprising predominantly C5 sugars with some C6 sugars, as well as byproducts of the pretreatment reaction that can inhibit yeast fermentation, most notably furfural, 5-hydroxy-methyl-furfural (5HMF), and acetic acid.

Hydrothermal pretreatment may be conducted at any pH level. In some embodiments, pretreatment may be conducted at pH > 8.0. In some embodiments, pretreatment is conducted at pH lower than 8.0, in some embodiments, between 2.5 and 8.0, or in some embodiments between

0.5 and 2.5, or in some embodiments between 2.5 and 5.0. In some embodiments, hydrothermal pretreatment is conducted without addition of acids, bases or other chemicals other than those derived from biomass processing steps.

Biomass feedstocks are typically pretreated to severity log Ro between about 3.3 and 4.5, or log Ro' between about -1.5 and 1.5. In some embodiments, biomass is pretreated at pH between 3.5 and 8.0 to severity Ro < 4.2, or less than 3.8, or greater than 3.4.

In some embodiments, after pretreated biomass is removed from a hydrothermal reactor, the liquid fraction and water insoluble solid component may be separated by means of solid/liquid separation methods known in the art. For example, solid/liquid separation may be achieved using a relatively inexpensive screw press, which can typically achieve total solids content of the solid residual to levels up to 45%. Alternatively a twin roll press or a twin wire press may be used, which can typically achieve total solids of the solid residual to levels in the range 30-45%. In some embodiments a belt press, drum filter, centrifuge, decanter centrifuge or other device known in the art may be used.

In some embodiments, where pretreated biomass is removed from a hydrothermal pretreatment reactor either by sluice system, by steam explosion, or by other techniques that provide pretreated biomass as a combined mixture of both "liquid fraction" component and water insoluble "fiber" component, this combined mixture can be directly used in enzymatic hydrolysis, with or without flashing or further dilution.

In some embodiments, unwashed water insoluble "fiber" component of pretreated biomass is used in enzymatic hydrolysis, with or without flashing or further dilution. As will be readily understood by one skilled in the art, "washing" of the water isoluble "fiber" component involves not merely dilution but exposure to a volume of water or aqueous solution that is not included in enzymatic hydrolysis

In some embodiments, pretreated biomass may be further subject to a de-lignification step, such as sodium hydroxide treatment or other methods known in the art.

Enzymatic hydrolysis of pretreated biomass, that is, enzymatic hydrolysis of cellulose to glucose and of hemicellulose to constituent oligo- and monosaccharides is typically conducted using a mixture of different hydrolytic enzyme activities. As is well known in the art, many fungi and bacteria have been identified which degrade lignocellulosic biomass in nature. Preparations of

enzymes obtained from such organisms typically provide a variety of different enzyme activities that, collectively and synergistically, provide effective hydrolysis of lignocellulosic materials. Such enzyme preparations are typically termed "cellulase" preparations, since they comprise cellulytic enzymes that catalyse hydrolysis of glucosidic linkages in cellulose, including endoglucanases, which introduce nicks in the cellulosic polymer chain thereby exposing reducing ends, and cellobiohydrolases or exoglucanases, which catalyze from reducing and non-reducing ends release of oligosaccharide products from the cellulosic polymer chain. Such preparations further typically comprise B-glucosidases, which catalyse hydrolysis of oligosaccharide products to fermentable monosaccharides as well as a variety of hemicellulases, including endoxylanases, exoxylanases, exoxylosidases, mannosidases, acetyl xylan esterases, and other activities. For review see (Rabinovich et al., 2002; Taherzadeh and Karimi, 2007).

A multi-enzyme mixture sufficient to hyrdrolyse pretreated biomass may be obtained by a variety of methods known in the art from a variety of microorganisms, including at least bacteria such as species of *Clostridium, Cellumonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Mocrobispora,* and *Streptomyces* and at least fungi such as species of *Trichoderma, Penicillium, Fusarium, Humicola, Aspergillus* and *Phanerochaete*. It will be readily understood by one skilled in the art that individual or groups of component enzyme activities may be isolated from such preparations and used separately, either in combination or as supplements to isolated multi-enzyme mixtures.

In practicing methods of the invention, an enzyme preparation is preferably used which has a substantially reduced susceptibility to glucose end-product inhibition of catalysis, meaning that the enzyme preparation exhibits less than 5% loss in absolute conversion yield due to the presence of 40 g/L glucose after 80 hours hydrolysis of wheat straw that has been hydrothermally pretreated to at least severity log Ro 3.5.

Pretreated EFB is first subject to partial initial hydrolysis, preferably using an enzyme preparation having substantially reduced susceptibility to glucose end-product inhibition of catalysis.

Suitable enzyme preparations that may be used to practice disclosed embodiments include commercially available cellulase preparations optimized for lignocellulosic biomass conversion. The term "commercially available cellulase preparation" refers to a mixture of enzyme activities that is sufficient to provide enzymatic hydrolysis of pretreated lignocellulosic biomass and that comprises cellulase, xylanase and B-glucosidase activities. The term "optimized for lignocellulosic

biomass conversion" refers to a product development process in which enzyme mixtures have been selected and modified for the specific purpose of improving hydrolysis yields and/or reducing enzyme consumption in hydrolysis of pretreated lignocellulosic biomass to fermentable sugars. Selection and modification of enzyme mixtures may include genetic engineering techniques, for example such as described in (Zhang et al., 2006) or by other methods known in the art. Commercially available cellulase preparations optimized for lignocellulosic biomass conversion are typically identified by the manufacturer and/or purveyor as such. These are typically distinct from commercially available cellulase preparations for general use or optimized for use in production of animal feed, food, textiles detergents or in the paper industry.

Any suitable commercially available cellulase preparation optimized for lignocellulosic biomass conversion may be used singly or in combination to practice the disclosed embodiments. Initial enzyme activity of such preparations typically comprises at least exoglucanases, endoglucanases, hemicellulases, and beta glucosidases. Such preparations typically comprise endoglucanase activity such that 1 FPU cellulase activity is associated with at least 31 CMC U endoglucanase activity and further typically comprise beta glucosidase activity such that 1 FPU cellulase activity is associated with at least at least 7 pNPG U beta glucosidase activity. It will be readily understood by one skilled in the art that CMC U refers to carboxymethycellulose units. One CMC U of activity liberates 1 umol of reducing sugars (expressed as glucose equivalents) in one minute under specific assay conditions of 50^{o} C and pH 4.8. It will be readily understood by one skilled in the art that pNPG U refers to pNPG units. One pNPG U of activity liberates 1 umol of nitrophenol per minute from para-nitrophenyl-B-D-glucopyranoside at 50^{o} C and pH 4.8. It will be further readily understood by one skilled in the art that FPU of "filter paper units" provides a measure of cellulase activity. As used herein, FPU refers to filter paper units as determined by the method of Adney, B. and Baker, J., Laboratory Analytical Procedure #006, "Measurement of cellulase activity", August 12, 1996, the USA National Renewable Energy Laboratory (NREL), which is expressly incorporated by reference herein in entirety.

Commercially available cellulase preparations optimized for lignocellulosic biomass conversion and provided by GENENCOR Tm may be used to practice disclosed embodiments. One specific example of such a cellulase preparation is sold under the tradename ACCELLERASE TRIO Tm.

Commercially available cellulase preparations optimized for lignocellulosic biomass conversion and provided by NOVOZYMES Tm may be used to practice disclosed embodiments. One specific example of such a cellulase preparation is sold under the tradename CELLIC CTEC3 Tm.

It will be readily understood by those of ordinary skill in the art that commercially available cellulase preparations optimized for lignocellulosic biomass conversion which become available in future will represent improvements over existing “generations” and will be at least as suitable for use in practicing methods oftnhe invention as are current “generations” of such preparations. It will be readily understood by those of ordinary skill in the art that commercially available cellulase preparations optimized for lignocellulosic biomass conversion may become available in future that are provided as individual components in which cellulytic enzyme activities are provided separately from xylanase and B-glucosidase activities with manufacturers' and/or purveyor's recommendations as to how these should be combined. Combinations of such separately provided preparations either according to manufacturer's recommendations or otherwise may also be construed as collectively forming a "commercially available cellulase preparation."

In some embodiments, it may be advantageous to supplement enzyme preparations with commercially available B-glucosidase preparations and/or xylanase preparations optimized for lignocellulosic biomass conversion. Commercially available xylanase preparations optimized for lignocellulosic biomass conversion and provided by NOVOZYMES may be used to practice disclosed embodiments. One specific example of such a xylanase preparation is sold under the tradename CELLIC HTEC2 Tm. Commercially available xylanase preparations optimized for lignocellulosic biomass conversion and provided by GENENCOR Tm may be used to practice disclosed embodiments. Two specific examples of such a xylanase preparation are sold under the tradename ACCELLERASE XY Tm and ACCELLERASE XC Tm. Commercially available B-glucosidase preparations provided by NOVOZYMES may be used to practice the disclosed embodiments. One specific example is the B-glucosidase preparation sold under the trade name NOVOZYMES 188.

In still other embodiments, it may be advantageous to supplement commercially available cellulase preparations optimized for lignocellulosic biomass conversion with B-glucosidase and/or xylanase preparations obtained from cellulytic microorgranisms. As is well known in the art, B-glucosidase helps reduce product inhibition of cellulose hydrolytic reactions, and, accordingly, it may be advantageous to supplement commercially available cellulase preparations optimized for lignocellulosic biomass with additional B-glucosidase activity. Further, some specific enzyme preparations, prepared by methods known in the art, have been reported to offer advantages as supplements to commercially available cellulase preparations optimized for lignocellulosic biomass conversion. See e.g. (Alvira et al., 2011).

Partial initial hydrolysis is stopped at incomplete conversion, in some embodiments at a point between 20 and 80% conversion, or between 30 and 70% conversion, or between 40 and 50% conversion. Following a solid/liquid separation step to recover the sugar streams from the initial hydrolysate, the solid residual fraction is resuspended in water or aqueous solution and the hydrolysis reaction allowed to proceed in a subsequent hydrolysis stage. Where a commercially available cellulase preparation optimized for lignocellulosic biomass conversion is used, the bulk of enzyme activity from initial partial hydrolysis can be simply transferred to subsequent hydrolysis because it will be associated with the solid residual. In some cellulase preparations, it may be advantageous to, optionally, supplement the subsequent hydrolysis reaction with B-glucosidase activity.

Partial initial hydrolysis may be conducted under a variety of conditions. In some embodiments, partial initial hydrolysis may be conducted at comparatively low total solids content, for example, at 10% total solids or lower. In some embodiments, partial initial hydrolysis may be conducted at higher total solids content, for example, greater than 10%, or greater than 18%, or greater than 19%, or greater than 20%. In other embodiments, partial initial hydrolysis may be conducted at total solids content between 20% and 40%, or between 40% and 60%. All numerical values presented here should be understood to apply to measured quantities with rounding to the number of significant digits shown. In some embodiments, partial initial hydrolysis is conducted using at least 1 kg of pretreated biomass, or at least 10 kg, or at least 100 kg, or at least 1000 kg.

One skilled in the art will be able to determine, through routine experimentation, an appropriate enzyme loading to use in partial initial hydrolysis, depending on the biomass feedstock used, the enzyme preparation used, and the total solids content at which partial initial hydrolysis is conducted.

Partial initial hydrolysis can be conducted to conversion levels of between 20 and 80%. In general, it is advantageous to stop partial initial hydrolysis at conversion levels associated with minimal loss of enzyme activity to the supernatant. As is well known in the art, enzyme preparations obtained from cellulytic organisms as well as commercially available cellulase preparations optimized for lignocellulosic biomass conversion will relatively quickly bind to lignocellulosic materials. With the exception of B-glucosidases and some specialized auxiliary enzymes, most of the component enzymes catalyse reactions on an undissolved surface, releasing a soluble reaction product. Most of the component enzymes are, accordingly, found associated with the insoluble "fiber" component

of pretreated biomass. As hydrolysis approaches complete conversion, however, a larger percentage of enzyme proteins are released into solution. Moreover, some particular enzymes from an enzyme mixture such as, for example, a commercially available cellulase preparation optimized for lignocellulosic biomass conversion, are released into solution earlier than others and some are never released at all. Accordingly, it is advantageous to stop partial initial hydrolysis at a stage where loss of enzyme proteins to solution is kept low, in order that no particular enzyme activity becomes "limiting" in subsequent hydrolysis.

In practicing the disclosed embodiments, it is advantageous to assess the extent to which enzyme proteins or measured enzyme activities of the enzyme preparations used are released to solution at different degrees of conversion and the hydrolysis times required to reach different degrees of conversion at the enzyme loading used in partial initial hydrolysis. It is advantageous to select a level of conversion at which to stop partial initial hydrolysis that results in minimal loss of enzyme proteins to solution, less than 10% loss, or less than 20% loss or less than 30% loss, or less than 33% loss, or less than 38% loss. Typically, using pretreated biomass that has not been subject to special de-lignification processes, loss of enzyme proteins to supernatant can be kept at levels of 33% loss or less up to at least 70% conversion. See e.g. (Gregg and Sadler, 1996; Tu et al 2009). Enzyme release to solution is generally increased where pretreated biomass has been subject to a de-lignification process.

It is generally advantageous to select a level of conversion that results in loss of less than 10%, or less than 20% of enzyme activity to solution. In this condition, transfer of enzymes to subsequent hydrolysis can be matinained at reasonable levels simply by transferring the solid residual recovered from partial initial hydrolysis, without requirement for any special effort to recover enzymes from the supernatant. Where loss of enzymes to supernatant during partial initial hydrolysis can be kept to levels beneath 10% or beneath 20%, enzyme activity can be recovered from subsequent hydrolysis steps at reasonable levels and recycled to other rounds of partial initial hydrolysis.

Some of the component enzymes of an appropriate enzyme mixture may differentially be released to solution at lower degrees of conversion than other component enzymes. Accordingly, it is advantageous to assess which components of the selected enzyme mixture are released to solution during partial initial hydrolysis, to determine whether supplementation of that component may be advisable in subsequent hydrolysis steps. For example, simple gel electrophoresis

methods well known in the art using solution samples obtained at different degrees of conversion can identify which protein bands are released first into solution.

In some embodiments, using commercially available cellulase preparations optimized for lignocellulosic biomass conversion, it is advantageous to maintain reactions temperatures at somewhat lower levels than those technically reported as "optimal" for enzyme activity, since these "optimal" levels may result in lowered stability of enzymes during long hydrolysis cycles, with resulting lowered recovery of enzyme activity in recycling and in transfer from partial initial hydrolysis to subsequent hyrdrolysis. For the commercially available cellulase preparations optimized for lignocellulosic biomass conversion and provided by GENENCOR Tm and NOVOZYMES Tm under the tradenames ACCELLERASE 1500 Tm and CELLIC CTEC2 Tm, reaction temperatures of 40-45o C typically provide the greatest long term stability with reasonable reaction yields. In practicing the disclosed embodiments, it is advantageous to compare long term stability of the selected enzyme preparation both at the suggested optimal temperature and also at some temperatures 5-10 degrees lower than the reported highest temperature value for optimum enzyme catalysis.

Partial initial hydrolysis can be stopped by a solid/liquid separation step, which produces both a liquid hydrolysate and a solid residual.

Solid/liquid separation after partial initial hydrolysis can be achieved using a variety of means well known in the art, including decanter centrifuges, belt presses, vacuum presses, screw presses, filter presses, drum dilters and other devices. In some embodiments, a solid/liquid separation technique is selected so as to provide optimal removal of suspended solids from the liquid hydrolysate, in order that subsequent fermentation processes may be conducted using well clarified solutions, which facilitate recycling of fermentive organisms.

The solid residual remaining after partial initial hydrolysis is then resuspended and subsequent hydrolysis conducted. The resuspension can be conducted using water, process solutions or buffers. In some embodiments, it can be advantageous to resuspend so as to achieve the highest practicable levels of total solids in subsequent hydrolysis. One skilled in the art will readily determine through routine experimentation an appropriate dilution in which to resuspend solid residual obtained from partial initial hydrolysis, depending on the pretreatment method and enzyme preparation selected. In some embodiments, a supplemental enzyme dose may be applied in a subsequent hydrolysis step. In some embodiments, two or more subsequent hydrolysis steps may

be conducted, with a solid/liquid separation between each step followed by resuspension and continued hydrolysis.

In some embodiments, the hydrolysate obtained after solid/liquid separation of the partial initial hydrolysis mixture, comprising C5 and C6 sugars and, optionally, comprising "fermentation inhibitors" and other pretreatment byproducts, can be directly fermented in a single fermentation reaction.

Comparisons between two-stage hydrolysis yields and one-stage hydrolysis yields are made by conducting one stage hydrolysis with equivalent pretreated EFB at lower dry matter content, by adding initially in the one-stage comparison an amount of water or aqueous solution corresponding to the amount of water added in the second hydrolysis stage. As is well known in the art, cellulose conversion can always be enhanced at equivalent enzyme dose by conducting hydrolysis at lower DM. Accordingly, as a control to evaluate the experimental effect of two-step hydrolysis, a single-step hydrolysis can be conducted having the same amount of biomass, but with an amount of water corresponding to the water used in both the first and second stages of a two-step hydrolysis. Samples can be analysed according to the method described by (Kristensen et al. 2009). The content of monosaccharides and disaccharides in the hydrolysed samples (D-glucose, D-xylose, L-arabinose, and D-cellobiose). The Conversion in absolute terms can be calculated as: $Cellulose\ conversion = \frac{g\ glucose/_{kg\ sample}}{g\ glucose\ potetiale/_{kg\ sample}}$

In some embodiments, conversion yields are improved at least 5%, or at least 6%, or at least 7%, or at least 8%, or at least 9%, or at least 10%, or at least 11%, or at least 12%, or at least 13% in absolute terms through use of multiple-stage hydrolysis, meaning that the hydrolysis yield in equivalent time and at equivalent enzyme dose is improved at least 5% in absolute terms over the corresponding yield obtained with equivalent EFB subject to hydrolysis in a single-stage hydrolysis to which is added a quantity of water equivalent to that used for re-suspension in subsequent hydrolysis stages.

In some embodiments, conversion yields are improved at least 5%, or at least 6%, or at least 7%, or at least 8%, or at least 9%, or at least 10%, or at least 11%, or at least 12%, or at least 13% in absolute terms through use of multiple-stage hydrolysis, meaning that the hydrolysis yield in equivalent time and at equivalent enzyme dose is improved at least 5% in absolute terms over the improved conversion that is achieved through relief from glucose product inhibition of cellulase

enzymes that is achieved by the two step hydrolysis, that is, improved in absolute terms by an amount, expressed as a percentage, corresponding to (two stage hydrolysis yield in absolute terms) - [(two stage hydrolysis in absolute terms obtained with equivalent EFB subject to hydrolysis in a two-stage hydrolysis to which is added in the second stage a quantity of glucose corresponding to the amount of glucose that is removed in the solid/liquid separation between the first and second hydrolysis stages) - (corresponding one stage hydrolysis yield in absolute terms with equivalent EFB)] / (one stage hydrolysis yield in absolute terms).

Examples.

1. Effect of washing the hydrothermal pretreated biomass before enzymatic hydrolysis.

Danish wheat straw was wetted to a DM of > 35% and pretreated at pH > 4.0 by steam to xylan number 10%, which corresponds to severity log Ro approximately 3.75. The pretreatment was conducted in the Inbicon pilot plant in Skærbæk, Denmark. The biomass was loaded into the pretreatment reactor (approx. 50 kg DM/h in continuous mode) using a sluice system and the pretreated biomass was removed from the reactor again using a similar sluice system. The pretreated biomass was subject to a washing step (agitator mixer with water addition of approx. 3 kg/kg DM) followed by a solid/liquid separation using a screw press, producing a liquid fraction and a solid fraction. The solid fraction had a DM content of about 30%, contained the majority of initial cellulose and lignin.

The washing degree of the produced material was varied by pressing the material in a screw press to a dry matter of approximately 60% followed be re-addition of various amounts of filtrate, Washing degree is defined as amount of dissolved solids removed.

EFB from Malaysia was pretreated at DM of > 35% and at pH > 4.0 by steam to xylan number 4-6%, which corresponds to severity log Ro approximately 4.0. The pretreatment was conducted in the Inbicon pilot plant in Skærbæk, Denmark. The biomass was loaded into the pretreatment reactor (approx. 50 kg DM/h in continuous mode) using a sluice system and the pretreated biomass was removed from the reactor again using a similar sluice system. The pretreated biomass was subject to a washing step (agitator mixer with water addition of approx. 3 kg/kg DM) followed by a solid/liquid separation using a screw press, producing a liquid fraction and a solid

fraction. The solid fraction had a DM content of about 30-40%, contained the majority of initial cellulose and lignin.

The washing degree of the produced material was varied by pressing the material in a screw press to a dry matter of approximately 60% followed by re-addition of various amounts of filtrate.

Solid fractions of washed pretreated wheat straw and EFB were subject to enzymatic hydrolysis as follows: Experiments were conducted in a 6-chamber free fall reactor working in principle as the 6-chamber reactor described and used in WO2006/056838. The 6-chamber hydrolysis reactor was designed in order to perform experiments with liquefaction and hydrolysis at solid concentrations above 20 % DM. The reactor consists of a horizontally placed drum divided into 6 separate chambers each 24 cm wide and 50 cm in height. A horizontal rotating shaft mounted with three paddles in each chamber is used for mixing/agitation. A 1.1 kW motor is used as drive and the rotational speed is adjustable within the range of 2.5 and 16.5 rpm. The direction of rotation is programmed to shift every second minute between clock and anti-clock wise. A water-filled heating jacket on the outside enables control of the temperature up to 80°C.

The chambers of the 6 chamber reactor were filled with app. 10 kg of pre-treated biomass adjusted to a dry matter content of 23,5 % WIS (water insoluble solids) for wheat straw and 14 % WIS for EFB. The pretreated biomass was then hydrolysed at 50°C and pH 5.0 to 5.3 using 0.033 ml Cellic CTec3™ from Novozymes / g glucan or 0.1 ml Accellerase TRIO™ from Dupont, Genecor / g glucan Enzymatic hydrolysis experiments were conducted for 24-120 hours at a mixing speed of 6 rpm.

Samples were analysed according to the method described by (Kristensen et al. 2009). The content of monosaccharides and disaccharides in the hydrolyzed samples (D-glucose, D-xylose, L-arabinose, and D-cellobiose) was quantified on a Dionex Ultimate 3000 HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80°C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1}. Samples were diluted with eluent and filtered through a 0.22 μm filter before analysis on HPLC.

Figure 1 shows the effect of washing degree (removed dissolved solids - DS) on hydrothermal pretreated wheat straw and EFB. The figure illustrates cellulose conversion as a function of washing efficiency measured as percentage removal of dissolved solids in the washing step between pretreatment and hydrolysis. There is a large positive effect on cellulose conversion by

increasing washing efficiency when using wheat straw. In contrast, with EFB, increasing washing efficiency does not affect cellulose conversion. In figure 1, the black line refers to Wheat straw 0.033 ml CTec3 / g glucan, 24 hours hydrolysis, 23.5 % SS; the gray line refers to EFB 0.1 ml Ac TRIO/ g glucan, 119 hours hydrolysis, 14 % SS.

2. Two-step hydrolysis of EFB.

EFB from Malaysia was pretreated at a DM of > 35% and pH > 4.0 by steam to xylan number 4-6%, which corresponds to severity log Ro approximately 4.0. The pretreatment was conducted in the Inbicon pilot plant in Skærbæk, Denmark. The biomass was loaded into the pretreatment reactor (approx. 50 kg DM/h in continuous mode) using a sluice system and the pretreated biomass was removed from the reactor again using a similar sluice system. The pretreated biomass was subject to a washing step (agitator mixer with water addition of approx. 3 kg/kg DM) followed by a solid/liquid separation using a screw press, producing a liquid fraction and a solid fraction. The solid fraction had a DM content of about 30-40% and contained the majority of initial cellulose and lignin. The degree of washing was found to be approximately 70%.

The two-step enzymatic hydrolysis of the solid fraction of washed pretreated EFB was conducted as follows: The first hydrolysis stage, or partial initial hydrolysis, was conducted in the 6 chamber reactor referred to in example 1. Chambers in the 6 chamber reactor were filled with 5.7 kg of pretreated EFB adjusted to 18% DM by addition of water. The pretreated biomass was then hydrolysed at 50°C and pH 5.0 to 5.3 using 0.1 ml Accellerase TRIO™ from Dupont, Genenncor / g glucan Enzymatic hydrolysis was conducted for 168 hours at a mixing speed of 6 rpm. After 96 hours of partial initial hydrolysis, a sample for use in subsequent hydrolysis was taken out of the chamber and separated by centrifugation and pressing of the pellet in a laboratory press. The separation resulted in a press cake with approx. 60% DM and a liquid phase. The press cake was re-suspended in 50 mM citrate buffer at pH 5.3 to a dry matter of 28% in a conical shake flask. pH was adjusted to 5.0-5.3 and the shake flask was incubated without further addition of enzyme on a shake table at 250 rpm at 50°C as the second hydrolysis stage.

As is well known in the art, cellulose conversion can always be enhanced at equivalent enzyme dose by conducting hydrolysis at lower DM. Accordingly, as a control to evaluate the experimental effect of two-step hydrolysis, a single-step hydrolysis was conducted having the same amount of biomass, but with an amount of water corresponding to the water used in both the first and second

stages of a two-step hydrolysis. Total dry matter in a two-step hydrolysis conducted as described would correspond to 15% DM in a single-step hydrolysis control.

The one-step enzymatic hydrolysis of the solid fraction of washed pretreated EFB was conducted as follows: The hydrolysis was conducted in the 6 chamber reactor referred to in example 1. Chambers in the 6 chamber reactor were filled with 5.7 kg of pretreated EFB adjusted to 15% DM. The pretreated biomass was then hydrolysed at 50°C and pH 5.0 to 5.3 using 0.1 or 0.22 ml Accellerase TRIO™ from Dupont, Genecor / g glucan Enzymatic hydrolysis was conducted for 144 hours at a mixing speed of 6 rpm.

Samples were analysed according to the method described by (Kristensen et al. 2009). The content of monosaccharides and disaccharides in the hydrolysed samples (D-glucose, D-xylose, L-arabinose, and D-cellobiose) was quantified on a Dionex Ultimate 3000 HPLC system equipped with a Shimadzu RI-detector. The separation was performed in a Phenomenex Rezex RHM column at 80 °C with 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1}. Samples were diluted with eluent and filtered through a 0.22 μm filter before analysis on HPLC. The Conversion was calculated as: $Cellulose\ conversion = \frac{g\ glucose / kg\ sample}{g\ glucose\ potetiale / kg\ sample}$

Results are shown in Figures 2a and 2b. In Figure 2a, the two gray lines show cellulose conversion as a function of time for one step hydrolysis with 0.1 ml Ac TRIO / g glucan enzyme, 15 % DM (triangles) and 0.22 ml Ac TRIO / g glucan enzyme, 15 % DM (stars). The black line shows cellulose conversion for a two-step hydrolysis: Black square: first hydrolysis, 0.1 ml Ac TRIO / g glucan, 18 % DM and Black circles: second hydrolysis without added enzyme, 28 % DM. The two-step hydrolysis shows a large enhancement of cellulose conversion relative to the lower DM control.

As shown, one step hydrolysis at 15% DM after 144 hours with a dose of 0.1 and 0.22 ml Accellerase Trio/g glucan respectively results in a cellulose conversion of 66 and 80% respectively. By performing a two-step hydrolysis at respectively 18 and 28 % DM, a cellulose conversion of 80% can be achieved using only 0.1 ml Accellerase Trio/g glucan in 168 hours of retention time. This illustrates that enzyme consumption can be reduced by a factor of 2x using two-step rather than single-step hydrolysis of hydrothermal pretreated EFB. It is further apparent that enzyme proteins are sufficiently retained by the residual solid fraction remaining after partial initial hydrolysis to support subsequent hydrolysis.

However, it can also be advantageous to proportion the enzyme dose so that it is administered in such manner that a supplemental enzyme dose is applied in the second hydrolysis stage, as shown in Figure 2b.

The gray line in Figure 2b shows cellulose conversion as a function of time for a one step hydrolysis; 0.16 ml Ac TRIO / g glucan; 16 % DM. The black line shows a two-step hydrolysis: Black square: first hydrolysis, 0.1 ml Ac TRIO / g glucan, 20 % DM and Black circles: second hydrolysis additional enzyme added 0.1 ml Ac TRIO / g remaining glucan, 27% DM. The DM and enzyme dose is equivalent overall for the one- and two-step hydrolysis reactions: 16 % DM and an enzyme dose of 0.16 ml Ac TRIO / g glucan.

As shown, one-step hydrolysis at 16% DM after 168 hours with a dose of 0.16 ml Accellerase Trio/g glucan resulted in a cellulose conversion of 78%. By performing a DM and enzyme equivalent two-step hydrolysis at respectively 20 and 27 % DM, a cellulose conversion of 92% can be achieved in equivalent hydrolysis time by adding 0.1 ml Accellerase Trio/g glucan in both steps. As 40 % of the glucan is removed in the solid/liquid separation the total enzyme dose for the two step hydrolysis is 0.16 ml Ac TRIO /g glucan.

3. Cellulase enzyme inhibition by filtrates obtained after first-step and second-step hydrolysis of EFB.

EFB from Malaysia was pretreated at a DM > 35% and a pH > 4.0 by steam to xylan number 4-6%, which corresponds to severity log Ro approximately 4.0. The pretreatment was conducted in the Inbicon pilot plant in Skærbæk, Denmark. The biomass was loaded into the pretreatment reactor (50 kg DM/h in continuous mode) using a sluice system and the pretreated biomass was removed from the reactor again using a similar sluice system. The pretreated biomass was subject to a washing step (agitator mixer with water addition of approx. 3 kg/kg DM) followed by a solid/liquid separation using a screw press, producing a liquid fraction and a solid fraction. The solid fraction had a DM content of about 30-40% and contained the majority of initial cellulose and lignin. The degree of washing was found to be approximately 70%.

Filtrate from the first hydrolysis step was produced as follows: The first hydrolysis stage, or partial initial hydrolysis, was conducted in the 6 chamber reactor referred to in example 1. A chamber in the 6 chamber reactor was filled with 10 kg of pretreated biomass comprising about 30-40 % DM

adjusted to 18% DM by addition of water. The pretreated biomass was then hydrolysed at 50°C and pH 5.0 to 5.3 using 0.1 ml Accellerase TRIO™ from Dupont, Genecor /g glucan Enzymatic hydrolysis was conducted for 96 hours at a mixing speed of 6 rpm. After 96 hours of partial initial hydrolysis, a sample for use in subsequent hydrolysis was taken out of the chamber and separated by centrifugation and the pellet was pressed in a laboratory press into a solid phase with approximately 60% DM and a liquid phase. This liquid phase was named H1 – liquid fraction after hydrolysis 1.

H1 was then tested for possible inhibitory effect on the cellulase enzyme preparation as follows: Pretreated EFB was diluted to 14% WIS with 50 mM citrate buffer pH 5.3 or H1 in a conical shake flask. The pretreated biomass was then hydrolyzed at 50°C and pH 5.0 to 5.3 using 0.16 ml Accellerase TRIO™ from Dupont, Genecor / g glucan Enzymatic hydrolysis was conducted for 48 hours at a mixing speed of 250 rpm in an incubation table.

Filtrate from the second hydrolysis step was produced as follows: The first hydrolysis stage, or partial initial hydrolysis, was conducted in the 6 chamber reactor referred to in example 1. A chamber in the 6 chamber reactor was filled with 10 kg of pretreated biomass comprising about 30-40% DM adjusted to 20% DM by adding water. The pretreated biomass was then hydrolysed at 50°C and pH 5.0 to 5.3 using 0.1 ml Accellerase TRIO™ from Dupont, Genecor /g glucan Partial initial hydrolysis was conducted for 71 hours at a mixing speed of 6 rpm. After 71 hours of partial initial hydrolysis, a sample for use in subsequent hydrolysis was taken out of the chamber and separated by centrifugation and the solid fraction was pressed in a laboratory press into a press cake with approximately 60% DM and a filtrate. The press cake was re-suspended in 50 mM citrate buffer with pH 5.3 to a dry matter of 27% in a conical shake flask, pH was adjusted to 5.0-5.3 and 0.1 ml Ac TRIO / g glucan was added. The shake flask was incubated on a shake table without further addition of enzyme at 250 rpm at 50°C for the second hydrolysis stage. After subsequent hydrolysis for 72 hours, the hydrolysate was separated by centrifugation and the pellet was pressed in a laboratory press into a press cake with approximately 60% DM and a liquid phase. This liquid phase is named H2 - filtrate after hydrolysis 2.

H2 was then tested for possible inhibitory effect on the cellulase enzyme preparation as follows: Pretreated EFB was diluted to 18% WIS with 50 mM citrate buffer pH 5.3 or H2 . The pretreated biomass was then hydrolysed in a conical shake flask at 50°C and pH 5.0 to 5.3 using 0.075 ml Accellerase TRIO™ from Dupont, Genecor /g glucan Enzymatic hydrolysis was conducted for 84 hours at a mixing speed of 250 rpm, 50°C in an incubation table.

Results are shown in Figures 3a and 3b. As shown in Figure 3a, when hydrolysis is conducted in buffer, 50 g/kg of glucose is released. However, when the pretreated EFB is diluted with H1 only 37.6 g/kg of glucose is released, where a concentration of 40.7 g/kg was measured from the beginning and a final concentration of 78.3 g glucose/kg was obtained. This indicates that some one or more compounds which came into solution during the course of the first hydrolysis step, which are present in H1, inhibit one or more component of the enzyme preparation or otherwise inhibit enzymatic hydrolysis.

As shown in Figure 3b, when hydrolysis is conducted in buffer, 30.7 g glucose/kg is released. When the pretreated EFB is diluted with H2, an equivalent amount of glucose, 30.1 g/kg, is released where a concentration of 40.0 g/kg was measured from the beginning and a final concentration of 70.9 was obtained. These results indicate that, as expected, the latest generation of commercially available cellulase preparations optimized for lignocellulosic biomass conversion, in this case ACCELLERASE TRIO Tm is not inhibited up to 40 g/kg glucose. These results further indicate that the compounds present in H1 which inhibit enzymatic hydrolysis are no longer present at inhibitor levels in H2. Without wishing to be bound by theory, these results are consistent with an interpretation that the inhibitory compounds in H1 are released during hydrolysis of the most readily-hydrolysed or external components of EFB fibers, such that inhibitory levels of these compounds are not achieved during the second hydrolysis stage. V

4. Determination of appropriate conversion target for partial initial hydrolysis.

It is advantageous to determine an appropriate target conversion level for partial initial hydrolysis, at which most enzyme proteins remain associated with the residual solid. The process described in this example using wheat straw can be repeated using EFB and later generation commercially available cellulase preparations optimized for lignocellulosic biomass conversion.

Wheat straw was soaked prior to pretreatment at 20-35% dry matter with aprox. 10 g acetic acid/kg dried weight biomass. Approx. 60 kg/h wheat straw or corn stover were pretreated at temperature about 185°C with a residence time of 12 minutes. Biomass was removed from the pretreatment reactor using a sluice device or "particle pump." The solid and liquid fraction of the pretreated biomass was afterwards separated by screw press resulting in a solid fraction having aproximately 30% total solids (including both insoluble fiber and dissolved solutes). The solid fraction was washed with 3 kg water/kg dry biomass and pressed again to 30% total solids.

Washed fiber was hydrolysed using a commercially available cellulase preparation optimized for lignocellulosic biomass conversion provided by NOVOZYMES Tm and provided under the tradename CELLIC CTEC2.

The release of enzyme protein to solution was measured at various time points.
Figure 4 shows the percentage of enzyme protein free in solution at different hydrolysis times corresponding to different degrees of conversion. As shown, using this enzyme preparation, enzyme protein loss to supernatant can be kept lower than 10% where partial initial hydrolysis is stopped at 60% conversion.

The descriptions and examples herein are representative only, and not intended to limit the scope of the claims.

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Claims.

1. A method of processing empty fruit bunches (EFB) to fermentable sugars comprising
- providing EFB feedstock,
- pre-treating the feedstock using hydrothermal pre-treatment,
- subjecting the pretreated feedstock to partial enzymatic hydrolysis using a cellulase preparation,
- separating the liquid hydrolysate from the solid residual obtained from partial hydrolysis, and
- resuspending the solid residual from partial hydrolysis and continuing enzymatic hydrolysis in a subsequent hydrolysis stage.

2. The method of claim 1 wherein a commercially available cellulase preparation optimized for lignocellulosic biomass conversion is used for enzymatic hydrolysis.

3. The method of claim 1 wherein the enzyme preparation used for enzymatic hydrolysis exhibits substantially reduced glucose end-product inhibition of catalysis.

4. The method of claim 2 wherein enzymatic hydrolysis in a subsequent hydrolysis stage is conducted using a supplemental dose of enzyme preparation.

5. The method of claim 1 wherein hydrothermal pretreatment is conducted to severity at least log Ro 3.8.

6. The method of claim 1 wherein hydrolysis conversion yield is improved in absolute terms by at least 5%.

7. The method of claim 1 wherein hydrolysis conversion yield is improved in absolute terms by at least 5%.

8. The method of claim 1 wherein hydrolysis conversion yield is improved at least 5% in absolute terms by an amount, expressed as a percentage, corresponding to (two stage hydrolysis yield in absolute terms) - [(two stage hydrolysis in absolute terms obtained with equivalent EFB subject to hydrolysis in a two-stage hydrolysis to which is added in the second stage a quantity of glucose corresponding to the amount of glucose that is removed in the solid/liquid separation between the

first and second hydrolysis stages) - (corresponding one stage hydrolysis yield in absolute terms with equivalent EFB)] / (one stage hydrolysis yield in absolute terms).

Figure 1

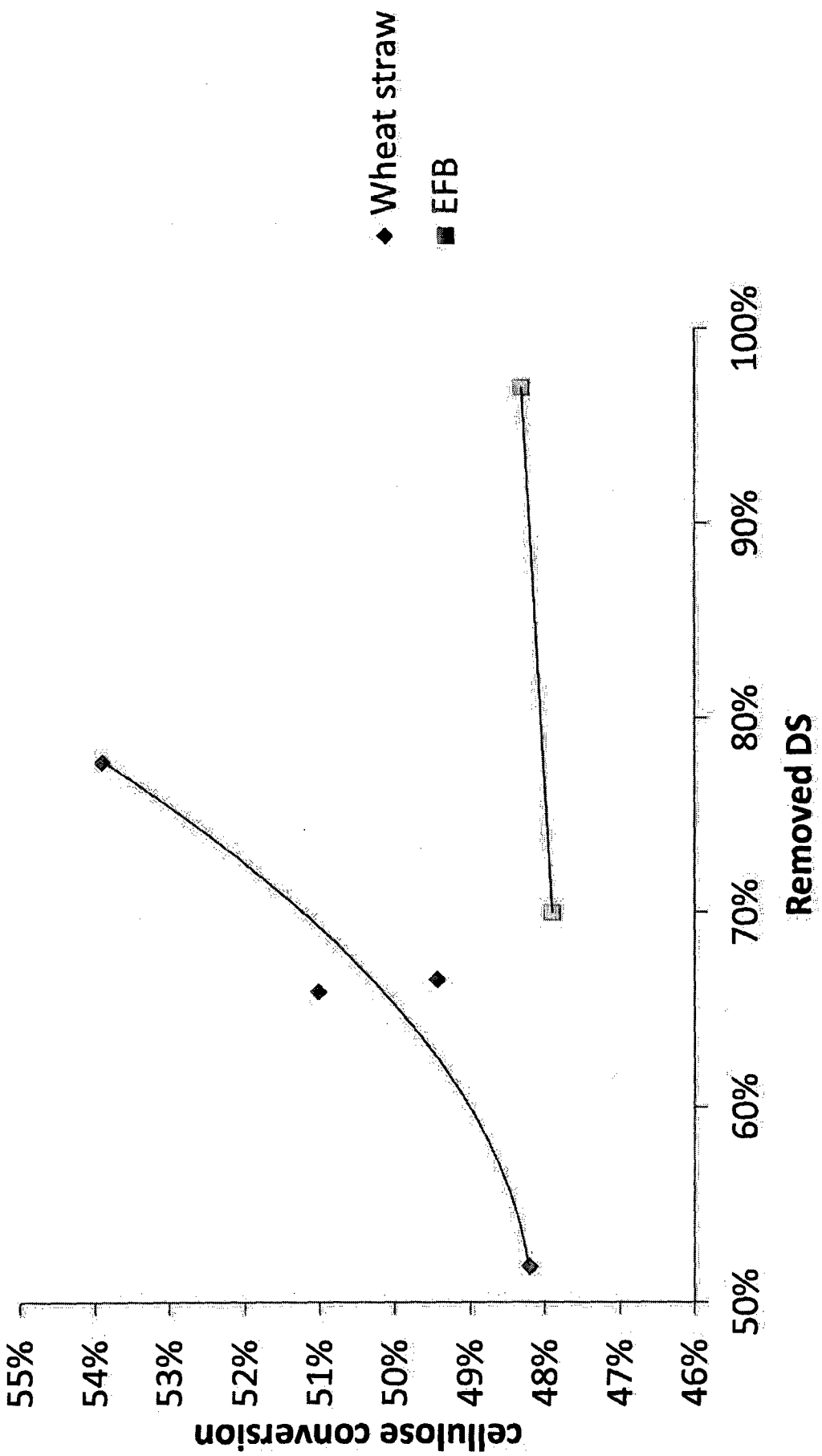

Wheat straw
EFB
cellulose conversion
55%
54%
53%
52%
51%
50%
49%
48%
47%
46%
50%
60%
70%
80%
90%
100%
Removed DS

Figure 2.a

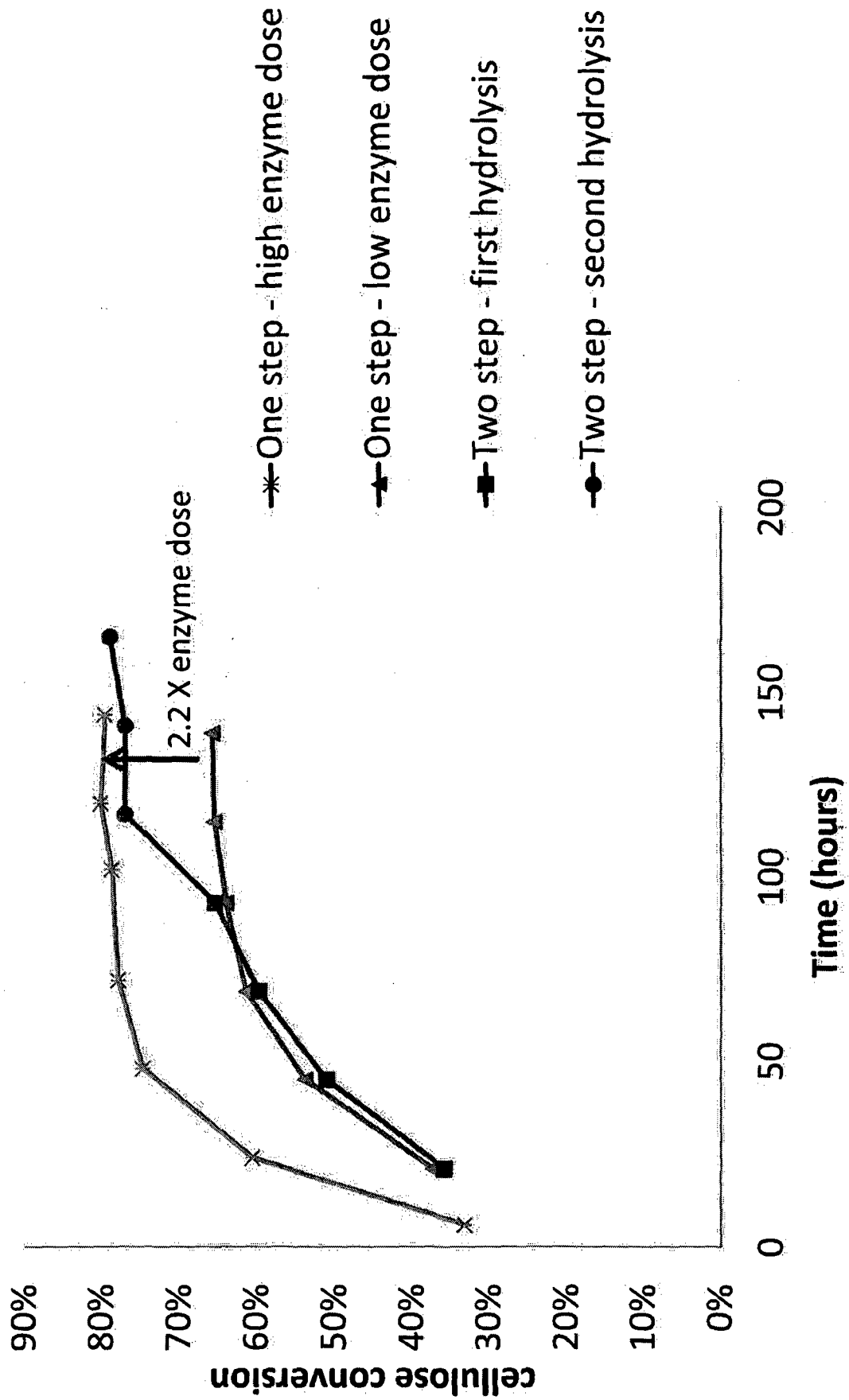

One step - high enzyme dose
One step - low enzyme dose
Two step - first hydrolysis
Two step - second hydrolysis
2.2 X enzyme dose
cellulose conversion
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
50
100
150
200
Time (hours)

Figure 2.b

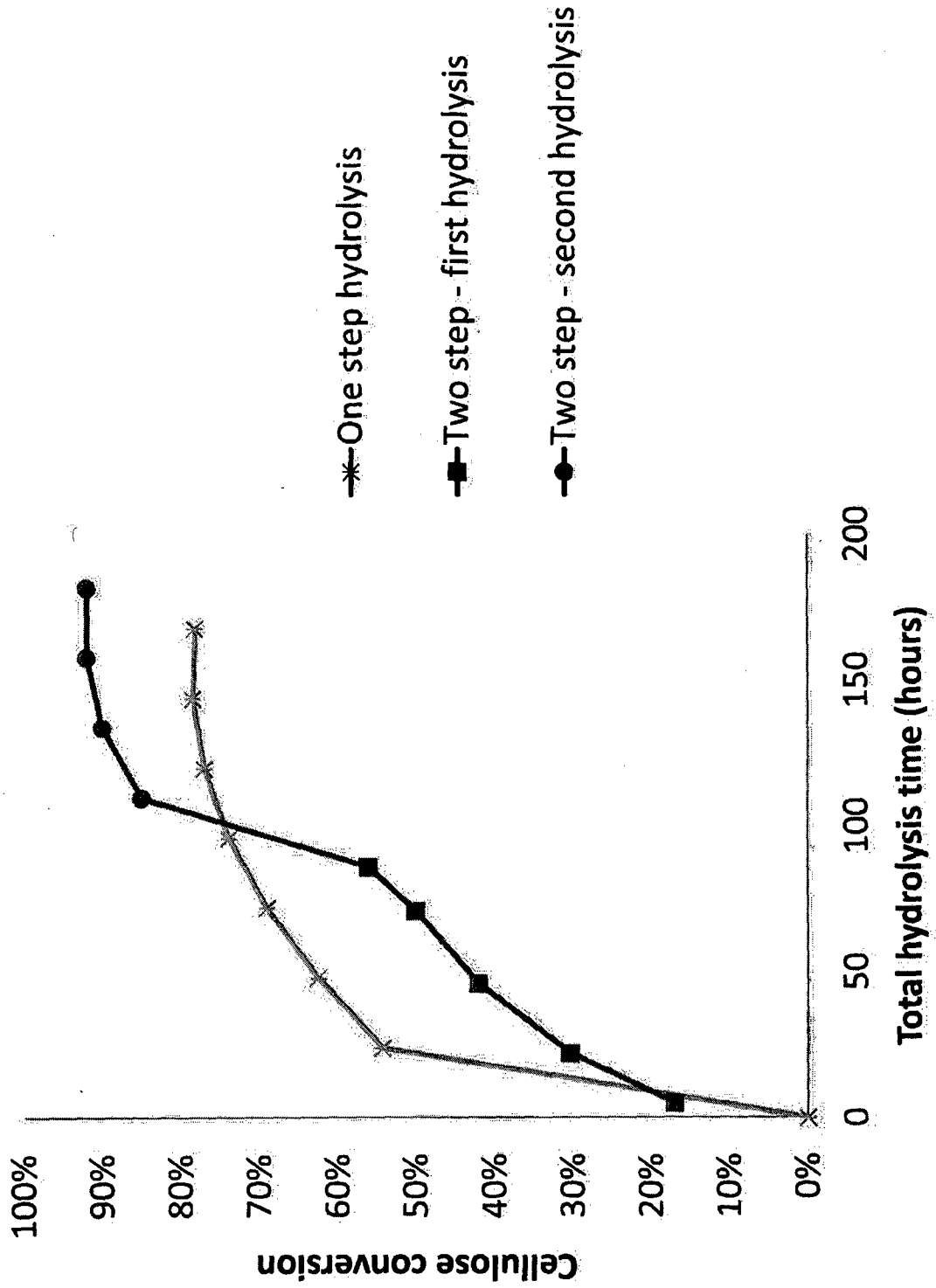

One step hydrolysis
Two step - first hydrolysis
Two step - second hydrolysis
Cellulose conversion
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
50
100
150
200
Total hydrolysis time (hours)

Figure 3a

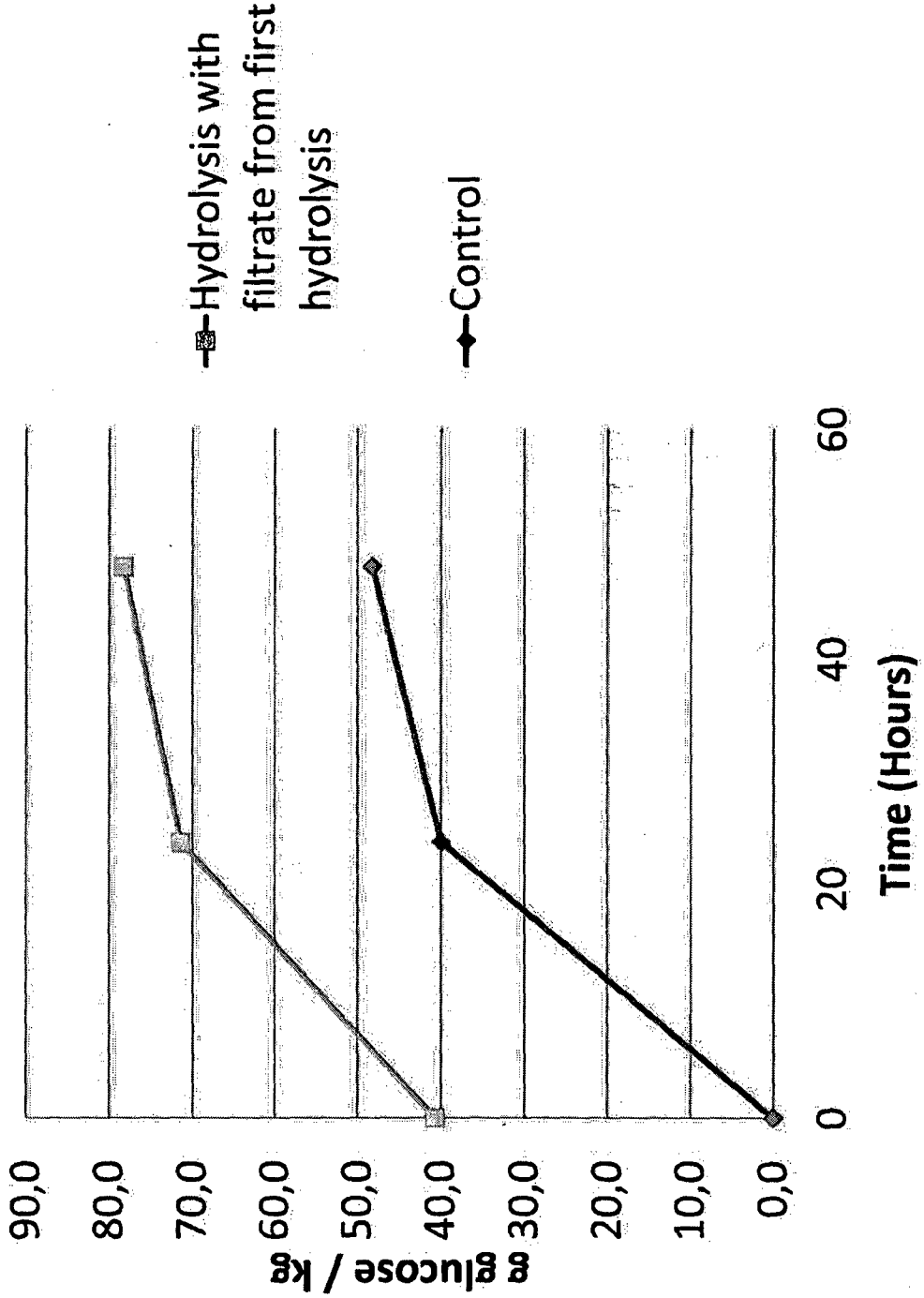
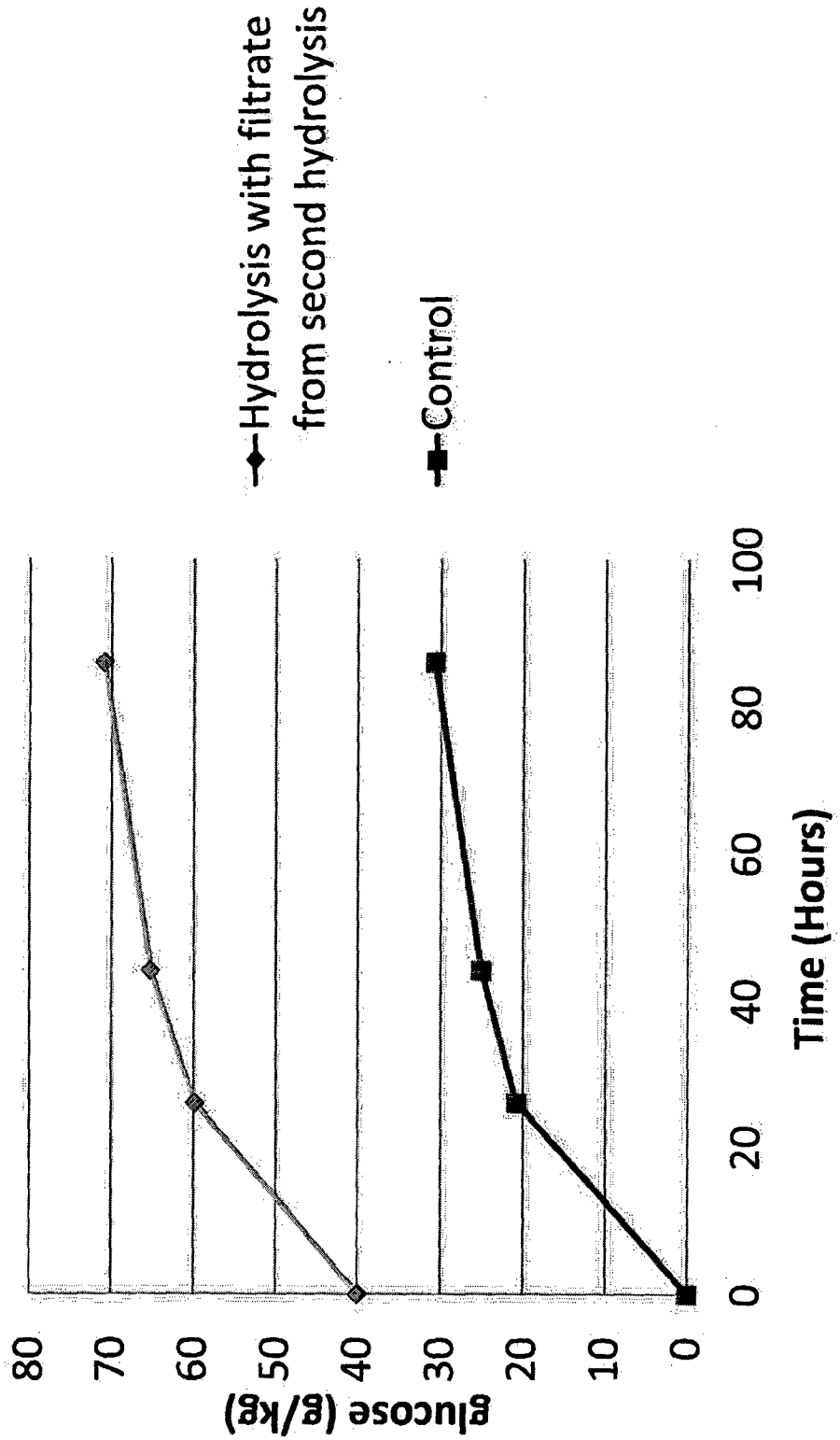

Hydrolysis with filtrate from first hydrolysis
Control
g glucose / kg
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
0
20
40
60
Time (Hours)

Figure 3b


Hydrolysis with filtrate from second hydrolysis
Control
glucose (g/kg)
0
10
20
30
40
50
60
70
80
Time (Hours)
0
20
40
60
80
100

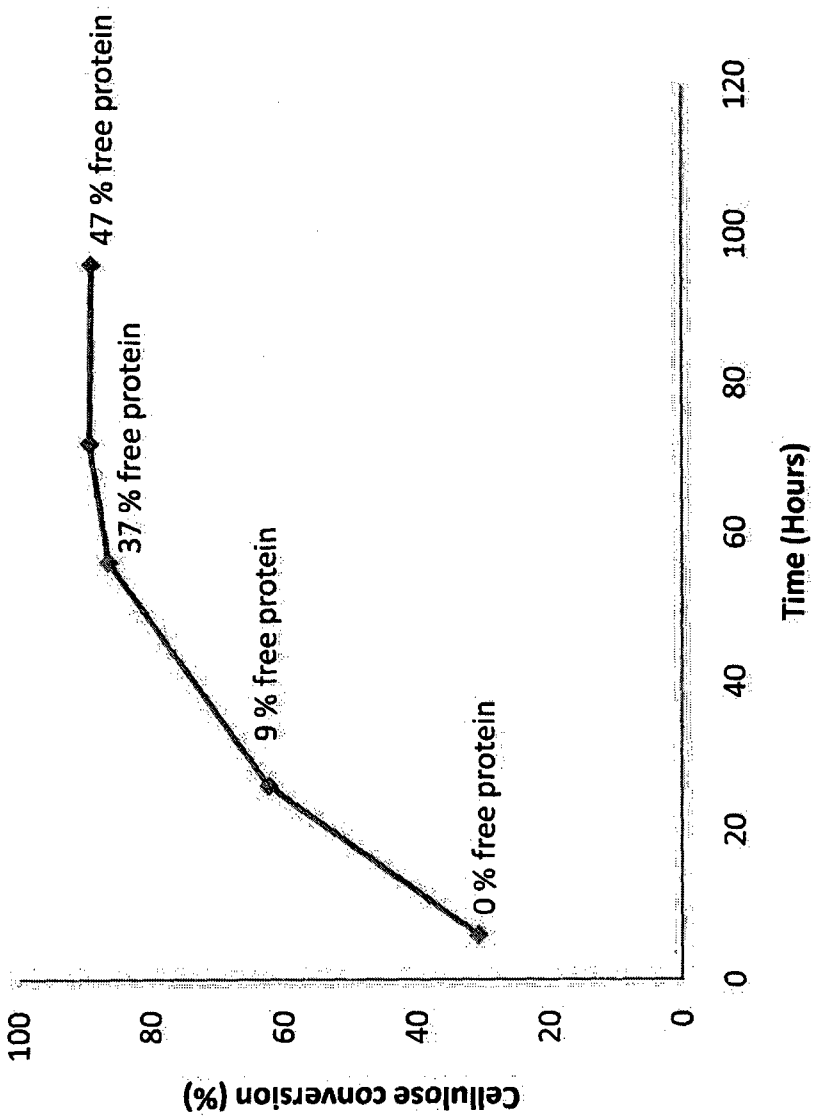


Figure 4. Percentage of enzyme protein free in solution at different hydrolysis times corresponding to different degrees of conversion.

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2013/050388

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/02 C12P19/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/147263 A1 (IOGEN ENERGY CORP [CA]; TOLAN JEFFREY S [CA]; WHITE THERESA [CA]; TOMA) 27 December 2007 (2007-12-27) the whole document & US 2010/068768 A1 18 March 2010 (2010-03-18) cited in the application ----- -/--	1-8

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2014	06/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Siatou, Evangelia

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2013/050388

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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